Molecular cloning of newt sex pheromone precursor cDNAs: evidence for the existence of species-specific forms of pheromones

T. Iwata[a], K. Umezawa[a], F. Toyoda[b], N. Takahashi[a], H. Matsukawa[a], K. Yamamoto[a], S. Miura[c], H. Hayashi[d], S. Kikuyama[a,*]

[a] *Department of Biology, School of Education, Waseda University, Nishi-waseda 1-6-1, Shinjuku-ku, Tokyo 169-8050, Japan*
[b] *Department of Physiology, Nara Medical University, Kashihara 634, Japan*
[c] *RI Center, School of Medicine, Yokohama City University, Yokohama 236, Japan*
[d] *Gunma Prefectural College of Health Science, Maebashi 371, Japan*

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Abstract Cloning of cDNA encoding a decapeptide pheromone (sodefrin) that attracts conspecific female newts was attempted. A cDNA clone encoding a protein consisting of 189 amino acid residues including a sodefrin sequence was isolated from a *Cynops pyrrhogaster* abdominal gland cDNA library. Likewise, a cDNA clone encoding a molecule comparable to the sodefrin precursor was obtained from a *Cynops ensicauda* abdominal gland cDNA library. This clone encoded a precursor protein of 192 amino acid residues, including a sodefrin-like peptide sequence with substitutions of two amino acid residues. This is the first report of a peptide pheromone precursor in vertebrates.

Key words: Female newt attracting pheromone; Sodefrin; Pheromone precursor protein; cDNA cloning

1. Introduction

It has been well recognized that courting males of the genera *Cynops* and *Triturus* attract sexually developed females by directing the water around the cloaca toward the female's snout by vibrating the tail, and that during courtship the male projects from the cloaca numerous minute tubules that are connected to the abdominal gland [1]. This suggests that the male newt emits female attracting pheromones from the cloaca. In fact, males of the newts *T. cristatus* and *C. pyrrhogaster* from which the abdominal glands have been removed are less able to attract females than intact males [2,3]. It has also been shown electrophysiologically that the olfactory mucosa of the *T. cristatus* female is considerably activated by extracts of the abdominal glands [4]. Thus, it has been considered that some chemical substances secreted by the abdominal gland stimulate the female through the olfactory system. Recently, we have purified and characterized a female attracting pheromone from the abdominal gland of the cloaca of *C. pyrrhogaster* [5]. This pheromone, designated sodefrin, is a decapeptide showing no sequence homology with any other known peptide. The fact that sodefrin consists of 10 amino acid residues prompted us to hypothesize the presence of a precursor molecule for sodefrin.

Sodefrin attracts sexually mature *C. pyrrhogaster* females but not females of a congeneric species, *C. ensicauda*, which are attracted to an aqueous extract of abdominal glands from males of their own species [5]. This indicates that a different form of female attracting pheromone, possibly a variant type of sodefrin, is present in the abdominal gland of *C. ensicauda*.

In this study, molecular cloning of a cDNA encoding sodefrin was attempted to demonstrate the presence of the pheromone precursor. A cDNA clone comparable to the sodefrin precursor clone was obtained from a cDNA library constructed from *C. ensicauda* abdominal gland mRNAs. The biological activity of the sodefrin-like peptide synthesized according to the amino acid sequence deduced from the nucleotide sequence of this cDNA was tested.

2. Materials and methods

2.1. Amplification of partial cDNA encoding sodefrin by polymerase chain reaction (PCR)

Total RNA (about 3 μg) was extracted from the abdominal glands of *C. pyrrhogaster* males using ISOGEN RNA extraction reagent (Nippon Gene). The mRNA was purified from the total RNA using oligo dT)-latex (Oligotex-dT30 super, TaKaRa), reverse-transcribed to cDNA, double-stranded and ligated with the *Eco*RI linker, followed by ligation into the *Eco*RI site of the vector, λgt11 (Stratagene), using a Timesaver cDNA synthesis kit (Pharmacia). Amplification of the partial cDNA encoding sodefrin by PCR was performed in *Ex-Taq* buffer, containing 0.2 mM each dNTP, 50 pmol of each synthetic degenerated primer, encoding the partial sodefrin sequence and the specific sequence of λgt11, respectively (sodefrin sense primer: 5′-AT (ACT) CC (AGCT) TC (AGCT) AA (AG) GA (CT) GC-3′; λgt11 antisense primer: 5′-GGTGGCGACGACTCCTGGAGCCCG-3′ or 5′-TTGACACCAGACCAACTGGTAATG-3′) with 0.5 units *Ex-Taq* polymerase (TaKaRa) per 20 μl reaction solution. The amplified cDNA was subcloned into the pT7-blue T-vector (Novagen). The plasmid containing the cDNA encoding sodefrin was used to transform a JM109 competent cell (Toyobo) and was subjected to sequencing analysis.

2.2. Isolation of cDNAs encoding sodefrin and a sodefrin-like peptide

C. pyrrhogaster and *C. ensicauda* cDNA libraries constructed using a Zap Express kit (Stratagene) were plated on four large (diameter 140 mm) LB agar plates with LB containing 0.7% agarose at a concentration of 2×10^4 pfu per plate. After being transferred to membrane filters, the cDNAs were denatured, followed by fixation to the membranes. Filters were pre-hybridized in a hybridization solution consisting of six-fold standard saline citrate (SSC), 0.2% (w/v) bovine serum albumin, 0.4% (w/v) Ficoll 400, 0.4% (w/v) polyvinylpyrrolidone and 1% (w/v) sodium dodecyl sulfate (SDS) at 50°C for 2 h. Hybridizations were performed with the radiolabeled partial cDNA encoding sodefrin (PCR product) employing the random priming method [6] in the same solution at 65°C for 16 h. The filters were washed twice in 0.1-fold SSC containing 1% SDS for 30 min at 65°C and placed in contact with an imaging plate by means of BAS-2000 II (FujiFilm) for 2 h. The clones giving positive signals were subcloned into a pBK-CMV plasmid vector using the Zap express kit and the sequences were analyzed by a cycle-sequence method, using a fluorescence-labeled primer with a Thermosequenase cycle sequencing kit (Amersham).

*Corresponding author. Fax: (81) (3) 3207-9694.
E-mail: kikuyama@mn.waseda.ac.jp

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The cDNAs were analyzed on a dNA sequencer model 4000L (LI-COR).

2.3. Northern blot analysis

Sodefrin and the sodefrin-like peptide precursor mRNAs were assessed by Northern blot analysis [7]. Total RNAs extracted from each tissue of both species of newts were electrophoresed in denaturing agarose gels (2.2 M formaldehyde) and transferred to a nylon membrane. The RNAs were fixed on the membrane by UV crosslinking. The membranes were pre-hybridized for 3 h at 60°C in hybridization solution. Hybridizations with the radiolabeled cDNAs were performed for 16 h at 60°C, adding the probe to the pre-hybridization solution. The cDNA was labeled by the random-priming method using an oligolabeling kit (TaKaRa) according to the manufacturer's instructions. The filters were washed with 0.1-fold SSC containing 0.1% SDS for 30 min at 65°C and placed in contact with X-ray film (Eastman Kodak) for 16 h at −80°C.

2.4. Biological test

The test for female attracting activity described by Toyoda et al. [3] was performed. Briefly, a plastic container (diameter 37 cm) was filled with 3000 ml of tap water and a female newt was kept in a cylinder (15 cm in diameter) placed in the center of the container. The container was divided into three sectors, into each of which a sponge block (5.6×7.3×3.4 cm) was gently placed. One block contained the test substance dissolved in 100 ml water, and the others contained tap water or a control substance. 30 s after introducing the sponge blocks, the inner cylinder was removed, the position of the test animal's snout was observed and the time spent by the snout in each sector was video-recorded for 10 min. In each series of tests, eight test females were used. The time spent by the snout of the test animals in each sector was analyzed statistically by Friedman's two-way analysis of variance, followed by the Wilcoxon matched-pairs signed-rank test.

3. Results

3.1. Isolation and characterization of cDNAs encoding sodefrin and the sodefrin-like peptide

Sequence analysis of PCR products obtained by using synthetic nucleotides coding a partial sodefrin sequence as a sense primer revealed that one clone 736 bp long contained the nucleotide sequence of the sodefrin molecule. Using this PCR product as a probe, sodefrin cDNA was screened from a *C. pyrrhogaster* abdominal gland cDNA library. The nucleotide sequence of the longest clone obtained was analyzed.

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                                   TGGCAGGTGAACAGGTGCAGAGACTCCATCACCCTATTCCTTACTCTCCTAGCACC   -1
ATGAGGGCCATCCTTGCAGCTGTCGTCCTGCTCCAGGCACTGATAACTGGAGATTGCCTATTATGCGAGCAGTGT   75
 M  R  A  I  L  A  A  V  V  L  L  Q  A  L  I  T  G  D  C  L  L  C  E  Q  C    25
TTCGCTCTCCAAACCAGCAGCTGCTCGGGTATCTTCACGCAGTGCTCTCCTGACGTCACTCACTGCGTCGCAGGC  150
 F  A  L  Q  T  S  S  C  S  G  I  F  T  Q  C  S  P  D  V  T  H  C  V  A  G    50
CTAGAGAACTGCACACTGGGGACTCATGTTATTCTAACTGCGTTCAAGGACTGTCTGGATCCTTCCGAAAAAGCA  225
 L  E  N  C  T  L  G  T  H  V  I  L  T  A  F  K  D  C  L  D  P  S  E  K  A    75
GCCTGCGGTAGAGAGGTCTCCTTCACAGCTCCAGCGGCCTCTTTATGGACAAGCAGGACGTGCTGTGACTCTGAT  300
 A  C  G  R  E  V  S  F  T  A  P  A  A  S  L  W  T  S  R  T  C  C  D  S  D   100
TTCTGCAACGGTGGGGATGTGCAGGTGCCTCCTCCAGACGACACTCCCAGTGGTTGTGGCAGTGACCAGCCCTGC  375
 F  C  N  G  G  D  V  Q  V  P  P  P  D  D  T  P  S  G  C  G  S  D  Q  P  C   125
ACAGCGCCAGAACACCTAAGGGAAACAGTGCACTCTACAACATCGATTAGAGAGAAGAGAAGAAAGCGATTTTTT  450
 T  A  P  E  H  L  R  E  T  V  H  S  T  T  S  I  R  E  K  R  R  K  R  F  F   150
TGGTCATATTTTCCGATCAGAAGAACGCATGTGGCACCATCTATGGAACTGCCTCCAGGCCGGCTAAGACTGGGG  525
 W  S  Y  F  P  I  R  R  T  H  V  A  P  S  M  E  L  P  P  G  R  L  R  L  G   175
AGGAGTATACCTTCAAAGGATGCACTACTCAAGATTTCTGCATAGCTGGAATTTTCCACATGGCGGGGATGCAAG  600
 R  S  I  P  S  K  D  A  L  L  K  I  S  A  *                                  189
CCTACGATTATTATGTTTTAAAGTGTTCCCCTGCCCTAAAAGTTTGAGACTTTTGTTCATACCCCATAGGCACTC  675
CTACTCTAGCTTAGTAGTTGTCTGTAGAAACATTCATAAAGCGCTACAAGTATGTGGAATGCAGTGTCTGATCTT  750
GTGATGAGGAAGCATATGAACTCATGTCAGCCTCTCTGAGACACAGTGTACAGGTGGCCAATGTGCTTAGTACAA  825
TCTAGGCCGGCATGCTGTTTAACCACTGTCTTCTCTATTCAGCCATCTTAAGCGCCTGGGCATCTCAGAGGGTTA  900
TCTTGGATTCATGCATCGAGTGATCCAAGCACAGGCCAAGCAATCATGCAATGATGCTGTCTTATGGTTGTAGAA  975
GGTGCTTCTCCTGATGTGCTACTAATGCTGACTTCATGAGTAGCCATGAACAGCCATTCCTGCTTTTCTTCTGCT 1050
TTTTGGTTGAATACCTCTTCTAACATAAAGTAATTGAGAATATCTGGCGCAGTTGTATTGATGCTGTCAAATATA 1125
AGAGGACAGGGTTATTGGTTCATATCTCCAATATGAATGTGCCTTTTAATCCAGCAATAAGCATGCTTTGTGCCA 1200
CAGCTATAACCCAAAATAGAACAAATATGTAGACCCGCTTTGTACTGCACATTGAAAAAATGAATAAACATTAAT 1275
TTACACTGCTGCAAAAAAAAAAAAAAAAAAAA                                            1308
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Fig. 1. Nucleotide and deduced amino acid sequences of the cDNA clone encoding sodefrin. The predicted amino acids are shown below the nucleotide sequence. The amino acid sequence contains an open reading frame encoding a 189 amino acid sodefrin precursor. The amino acid sequence of sodefrin is indicated by bold type. An asterisk indicates the termination codon. A polyadenylation signal (AATAAA) is underlined.

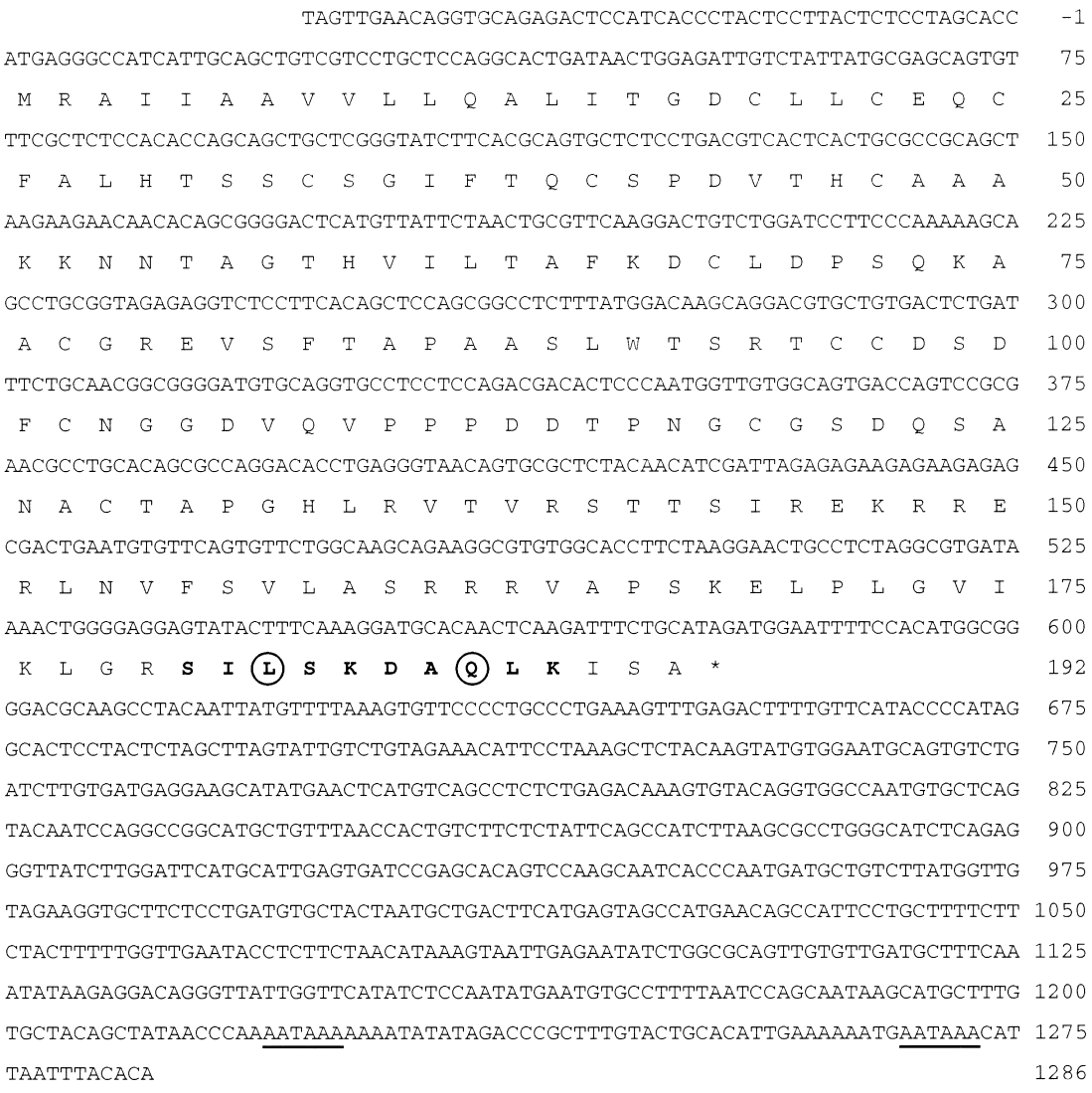

Fig. 2. Nucleotide and deduced amino acid sequences of cDNA clone encoding the sodefrin-like peptide. The predicted amino acids are shown below the nucleotide sequence. The amino acid sequence contains an open reading frame encoding a 192 amino acid precursor of the sodefrin-like peptide. The amino acid sequence of sodefrin-like peptide is indicated by bold type. An asterisk indicates the termination codon. Two polyadenylation signals (AATAAA) are underlined. The amino acid residues that differ from those of sodefrin are circled.

The clone was estimated to be 1364 bp long, with an open reading frame of 567 bp encoding a putative sodefrin precursor protein (Fig. 1). The sodefrin sequence (Ser-Ile-Pro-Ser-Lys-Asp-Ala-Leu-Leu-Lys) was located close to the C-terminus. This sodefrin precursor protein contained a putative hydrophobic signal peptide. The 5′ and 3′ untranslated regions were 56 and 741 bp long, respectively. A putative AATAAA polyadenylation signal was found 19 bp upstream of the poly(A)-tail. The molecular mass of the sodefrin precursor deduced from its nucleotide sequence was estimated to be about 20 kDa.

A cDNA encoding the sodefrin-like peptide was isolated from a *C. ensicauda* abdominal gland cDNA library using the sodefrin precursor cDNA as a probe (Fig. 2). The sequence of the longest positive clone was analyzed extensively. This clone consisted of 1339 bp and contained a predicted open reading frame of 576 bp encoding a precursor protein of 192 amino acid residues, including a series of putative hydrophobic amino acids that was presumed to be a signal peptide. The 5′ and 3′ untranslated regions were estimated to be 53 and 710 bp long, respectively. The nucleotide sequence encoding the predicted sodefrin-like peptide located in the region 9–39 bp upstream from the termination codon differed by only two bases from that encoding the sodefrin molecule. The deduced amino acid sequence of this sodefrin-like peptide was Ser-Ile-Leu-Ser-Lys-Asp-Ala-Gln-Leu-Lys, with two amino acid substitutions, Pro^3 by Leu, and Leu^8 by Gln, compared with the sodefrin sequence. The nucleotide sequence homology of the clone with pre-prosodefrin cDNA was 93.5% and the amino acid sequence deduced from the predicted open reading frame showed 81.9% homology with the corresponding region of the sodefrin precursor.

3.2. Northern blot analysis of total RNAs from various organs

The expression of sodefrin precursor mRNA was examined in different organs by Northern blot analysis using cDNA encoding the sodefrin precursor as a probe. It was revealed that sodefrin precursor mRNA was 1.5 kb long and was ex-

pressed in the abdominal gland but not in the other organs such as the tail, liver, kidney, testis and brain (Fig. 3a). Likewise the expression of sodefrin-like peptide precursor mRNA of *C. ensicauda* was also analyzed with the sodefrin-like peptide precursor cDNA as a probe. A single band showing positive hybridization was detected at a position corresponding to approximately 1.5 kb exclusively in the abdominal gland (Fig. 3b).

3.3. Female attracting activity of sodefrin-like peptide

The female attracting activity of the synthetic sodefrin-like peptide was tested using *C. ensicauda* and *C. pyrrhogaster* females as test animals. The sodefrin-like peptide attracted females of *C. ensicauda* but not those of *C. pyrrhogaster*, whereas sodefrin attracted females of *C. pyrrhogaster* but not those of *C. ensicauda* (Fig. 4a). The responses of *C. ensicauda* females were dependent on the amount of the peptide (1–100 ng) which was contained in the sponge block as a test substance, 10 ng being the minimum amount that exerted an effect (Fig. 4b).

4. Discussion

It has been predicted that the abdominal glands of *C. ensicauda* do not contain sodefrin, as an aqueous extract of these glands neither attracted *C. pyrrhogaster* females [5] nor contained substances that immunoreacted with an anti-sodefrin antiserum [8]. The results obtained in the present experiments raise the possibility that the *C. ensicauda* abdominal gland secretes a sodefrin-like pheromone. This is supported by the recent demonstration that abdominal gland extract of *C. ensicauda* contains a peptide exhibiting the same elution profile on HPLC as the synthetic sodefrin-like peptide (unpublished). The minimum effective amount of the synthetic sodefrin-like peptide that attracted *C. ensicauda* females, as determined by bioassay, was in good accord with that of sodefrin which attracted *C. pyrrhogaster* females [5]. The sodefrin-like peptide, like sodefrin, attracts only conspecific females. Minute amino acid sequence differences among species seem to determine species-specific activity and may be important for ensuring reproductive isolation.

Sequence analysis of the cDNAs encoding sodefrin and the sodefrin-like peptide revealed that both peptides are generated from precursor proteins. Both molecules are sandwiched between monobasic amino acid (Arg and Lys). It is well known that most of the peptide hormones are generally derived from precursor proteins as a result of processing. The peptide hormone sequences are sandwiched between a pair of dibasic amino acids that are subjected to cleavage with enzymes such as PC1 and PC2 [9]. The absence of such dibasic amino acids at the front and rear of sodefrin and the sodefrin-like peptide molecules suggests that the mode of processing of this peptide pheromone is different from that of ordinary peptide hormones. It has become evident, however, that cleavage at single basic residues also occurs in some cases of processing of peptide hormones [10]. Somatostatin-28 and somatostatin-14 are generated from a common precursor and released by endoproteolytic activation; a single arginine residue constitutes

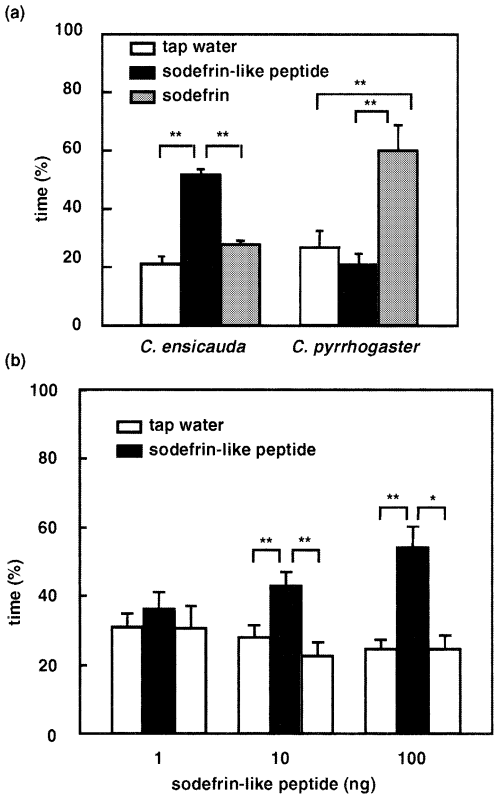

Fig. 4. a: Attracting effect of sodefrin and the sodefrin-like peptide on females of *C. ensicauda* and *C. pyrrhogaster*. Each sponge block contained tap water and 10 ng of sodefrin or sodefrin-like peptide. The preference tests were carried out as described in Section 2. Each column and vertical bar represent the mean of eight tests and S.E.M., respectively. $^{**}P < 0.01$. b: Dose-dependent responses of *C. ensicauda* females to sodefrin-like peptide (1–100 ng) contained in a sponge block. Each column and vertical bar represent the mean of eight determinations and S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$.

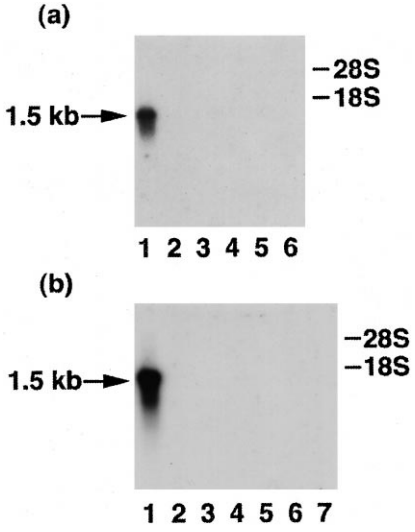

Fig. 3. a: Northern blot analysis of total RNA, isolated from various organs of *C. pyrrhogaster*. Total RNAs (5 μg) of the abdominal gland (lane 1), brain (lane 2), testis (lane 3), kidney (lane 4), liver (lane 5) and tail (lane 6) were hybridized with radiolabeled sodefrin precursor cDNA. b: Northern blot analysis of total RNA from various organs of *C. ensicauda*. Total RNAs (5 μg) of the abdominal gland (lane 1), brain (lane 2), testis (lane 3), kidney (lane 4), liver (lane 5), tail (lane 6) and intestine (lane 7) were hybridized with the radiolabeled sodefrin-like peptide precursor cDNA.

the cleavage site for the release of somatostatin-28, whereas proteolytic cleavage at a dibasic sequence (Arg-Lys) generates somatostatin-14 [11,12]. Mackin et al. [13,14] purified two candidate somatostatin convertases from anglerfish secretory granules capable of generating somatostatin-14 and somatostatin-28. It has been confirmed that synthetic sodefrin C-terminally extended with isoleucine, serine and alanine (C-terminal portion of sodefrin precursor consisting of 13 amino acid residues) does not attract the females, indicating that these three amino acid residues must be removed from the C-terminus of this molecule to acquire biological activity. However, it remains to be clarified by what mechanisms these two pheromonal peptides are generated from the precursor proteins.

Northern blot analysis revealed that the length of sodefrin and the sodefrin-like peptide precursor mRNAs is about 1.5 kb. In the present experiment, the longest clones isolated from the libraries constructed from *C. pyrrhogaster* and *C. ensicauda* abdominal gland mRNAs were 1364 bp and 1339 bp, respectively. This suggests that a longer poly(A)-tail and/or 5′ untranslated region in front of the initiation codon is present in the mRNAs. Moreover, these pheromone mRNAs are expressed only in the abdominal gland so far studied.

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